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GRANT NUMBER: DAMD17-94-J-4106

TITLE: Alternative Splicing in Normal Development and in Breast Cancer

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REPORT DATE: July 1996

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1996	Final (1 Jul 94 - 30 Jun 96)

4. TITLE AND SUBTITLE
Alternative Splicing in Normal Development and in Breast Cancer

5. FUNDING NUMBERS
DAMD17-94-J-4106

6. AUTHOR(S)
John R. Bermingham, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California, San Diego
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Alterations in the splicing patterns of key regulatory genes are likely to play an important role in oncogenesis. The ASF/SF2 protein is one of a family of SR splicing factors that have been shown to regulate splice site choice *in vitro*. We have mapped the ASF/SF2 gene to 17q21.3-q22 in humans, and close to the *Ovum mutant* locus on chromosome 11 in mice. Our current objective is to examine the role of the ASF/SF2 gene in development and oncogenesis by observing the effects of disrupting the gene in mice. We were unable to achieve germ line transmission using embryonic stem cells that were heterozygous for an ASF/SF2 deletion. Highly chimeric mice were runted, possessed hypotrophic testes, and were sterile. Because of the possibility that this result is due to haploinsufficiency of the ASF/SF2 gene, and in light of new data that suggest that ASF/SF2 may be required for cell viability, we are constructing an inducible knockout vector for the ASF/SF2 gene.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer Alternative splicing, ASF/SF2, gene mapping, targeted mutagenesis	20
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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J. R. Bennett 7/30/90

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4. Table of Contents.

5. Introduction.

Alternative mRNA splicing plays an important role in development and differentiation; many transcripts are spliced differently in distinct cell types and tissues (for reviews see Smith et al., 1989; McKeown, 1992; Hodges and Bernstein, 1994). Many alternatively spliced mRNAs encode proteins that act in transcriptional regulation, in signal transduction, and in the contacts of cells with each other and with the extracellular matrix. Neoplastic growth can result from alteration of each of these processes, and for several genes that function in the development and differentiation of cells and tissues, novel splice variants have been identified from tumors. Transcripts that are differently spliced in tumors include mRNAs encoding fibronectin (Oyama et al., 1989; Oyama et al., 1990), estrogen receptor (reviewed in McGuire et al., 1991), and the cell suface glycoprotein CD44 (reviewed in Cooper, 1995; Ruiz et al., 1995). The observation that differently spliced mRNAs exist for these genes in cancer suggests that alteration(s) in the splicing machinery is involved in one or more stages in oncogenesis. An understanding of the relationship between alternative splicing and oncogenesis may have profound implications for the prevention, diagnosis and treatment of many cancers, including breast cancer, and may provide insights into normal development as well.

While examples of alternatively spliced transcripts abound, relatively little is known about the mechanisms involved in regulating the use of alternative splice sites. Both constitutive and alternative splicing occurs on spliceosomes, which are complex particles composed of small nuclear ribonucleoproteins, or snRNPs, and non-snRNP proteins (reviewed in Moore et al., 1993; Newman, 1994). The SR family of non-snRNP splicing factors are characterized by the presence of an RNA recognition motif and a serine and arginine rich (SR) domain (reviewed in Norton, 1994; Horowitz and Krainer, 1994; Fu, 1995). SR proteins are required at early stages of spliceosome assembly, have distinct but overlapping specificities for different pre-mRNAs, and can alter splice site choice (reviewed in Norton, 1994; Horowitz and Krainer, 1994; Fu, 1995). These observations suggest that SR proteins are involved in the regulation of alternative splicing *in vivo*. One of the best characterized SR proteins, ASF/SF2, is the focus of this report.

I am taking two experimental approaches to understand better the function of ASF/SF2 *in vivo*. First, I have mapped the gene in mice and humans, to assess potential associations of the gene with classical mouse mutations or human genetic diseases. Second, I am attempting to generate a mice that carry mutant alleles of the ASF/SF2 gene, to determine if it is required for normal or abnormal development, and to examine the effects of deletion of ASF/SF2 on patterns of RNA processing.

6. Body.

A. Chromosomal mapping of SR protein genes.

The first specific aim of this research project was to complete the mapping of the ASF/SF2 gene. This objective has been accomplished, and a paper describing the results has been published (Bermingham, et al., 1995). Initial mapping of the ASF/SF2 gene in humans to 17q22 raised the possiblity that it could be a candidate gene for the BRCA1 locus. While the ASF/SF2 gene lies near the BRCA1 locus, cloning of the BRCA1 gene (Miki et al., 1994), demonstrates that ASF/SF2 and BRCA1 are distinct genes. Interstitial deletions of chromosome 17 have been observed in some breast tumors (Cropp et. al., 1993). The loss of a copy of the ASF/SF2 gene could play a role in tumorigenesis if it led to the aberrant splicing of pre-mRNAs from BRCA1 or other gene(s) that function in growth control.

In mice, we have mapped the ASF/SF2 gene to mouse chromosome 11, near the marker D11Mit38 (Bermingham et al., 1995). These results indicate that the ASF/SF2 gene is very close to the *Ovum mutant* (*Om*) locus. *Om* is responsible for a genetic behavior of the DDK inbred mouse strain: DDK females are fertile when mated to DDK males, but are sterile or semi-sterile when mated to males of other inbred strains; DDK males are fertile when mated to females of other inbred strains (Wakasugi, 1974). Analysis of backcross mice (Sapienza et al., 1992) and recombinant inbred mice (Baldacci et al., 1992) placed the *Om* locus on mouse chromosome 11. ASF/SF2 was mapped using backcross mice between the C57BL/6 and DDK strains that were previously described in Sapienza et al., (1992) to locate ASF/SF2 more precisely, and to determine if it could be a candidate gene for the *Om* locus. These results are summarized in figure 1, and do not exclude ASF/SF2 as a candidate gene for *Om*, although recent high resolution mapping of the *Om* locus (Baldacci et al., 1996) indicates that it lies proximal to the ASF/SF2 gene. Because of the proximity of the Sfrs1 and *Om* loci, and because the effect of the *Om* locus has been previously hypothesized to result from a reversal of imprinting at the *Om* locus in the DDK strain (Sapienza et al., 1992), we hypothesized that the ASF/SF2 gene may be imprinted, as are the genes that encode two other splicing factors, snRNP associated protein N, and U2afbp-rs (Leff et al., 1992; Hayashizaki et al., 1994). Genomic imprinting is a little-understood phenomenon that may play important roles in normal development, as well as the etiology of some tumors (for reviews, see Tycko, 1994; Efstratiadis, 1994). However, the results of recent genetic experiments are inconsistent with imprinting of *Om* (Pardo-Manuel de Villena et al., 1996), and therefore there is currently no reason to believe that the ASF/SF2 gene is imprinted.

B. Targeted mutagenesis of the ASF/SF2 gene.

The basic procedures for generating mutations in mice by homologous recombination are extensively documented (Joyner, 1993; Wassarman and DePamphilis, 1993; Hogan et al., 1994). Briefly, the procedure consists of the following steps: 1) A targeting construct is built that will undergo homologous recombination with the gene to be disrupted. 2) Mutations are produced in embryonic stem (ES) cells in culture by homologous recombination of the target gene with the introduced DNA. 3) Mutant ES cells are introduced into host blastocysts to that are in turn introduced into pseudopregnant mice and allowed to develop into chimeric mice. 4) The chimeric mice are bred to determine if the ES cells contribute to the germ line of the chimera. If so, mutations that they carry can be transmitted to future generations.

Figure 2 diagrams the knockout construct that was made to disrupt the ASF/SF2 gene, and Table 1 summarizes the progress made so far in producing mice that carry a disrupted ASF/SF2 allele. I have generated seven new ES cell lines that are heterozygous for a deletion of the ASF/SF2 gene. Five of these cell lines have been injected into blastocysts and implanted into pseudopregnant mice. Chimeric mice have been obtained from four of the five cell lines. None of these mice produced ES cell-derived progeny. Highly chimeric mice were runted, possessed hypotrophic testes in which germ cells were absent (Figure 3), and therefore were sterile. The failure to obtain germline transmission of the ASF/SF2 mutation could result from technical problem(s) that could be solved with additional blastocyst injections, perhaps with new heterozygous ES cell lines. However, ES cells with a mutation in a different gene that were selected in parallel to those that possess the ASF/SF2 mutation did permit germline transmission. Alternatively, the ASF/SF2 gene may be haploinsufficient in one or more cell types that results in the failure of germ cell proliferation in the testes. Currently, we cannot distinguish between these possiblities. I have chosen to construct a new knockout vector that will permit a funtional ASF/SF2 gene to be deleted in mice by tissue-specific expression of the Cre site-specific recombinase. This approach was undertaken for two reasons. First, deletion of ASF/SF2 in a chicken B-cell line (Wang and Manley, 1996) suggest that the gene is essential for cell viability. Both copies of the chicken gene can be deleted only in the presence of a stably integrated human ASF/SF2 expression vector, indicating that in this cell type, and perhaps many others, deletion of the ASF/SF2 gene is lethal to the cell. To determine the extent of ASF/SF2 expression in embryos, an *in situ* hybridization probe for ASF/SF2 was constructed (Figure 4). Hybridization of this probe to E11.5 and E14.5 embryos revealed widespread expression of ASF/SF2 at both times in development. Together, these results suggest that the absence of ASF/SF2 in all cells is likely to result in early embryonic lethality. Deletion of ASF/SF2 only in specific cells will permit most of development to proceed normally, while allowing the effects

of the deletion on differentiation and RNA processing patterns to be examined.

The steps involved in tissue-specific gene deletion are as follows (Figure 5; for review, see Chambers, 1994):

1. A knockout vector is constructed that contains three loxP sites. Cre recombinase catalyzes site-specific recombination between these sites. An essential part of the gene of interest is flanked by two loxP sites, while a third loxP site flanks the Tk and neo selectable marker genes.

2. Homologous recombination between the endogenous gene and the knockout vector render ES cells resistant to neomycin, while the function of the gene of interest is maintained.

3. Transient expression of Cre recombinase in ES cells excises either the neo and TK selectable markers (type II recombination) or both the selectable markers and sequences from the gene of interest (type I recombination). Recombinant ES cells that lack the TK gene are selected for using gancyclovir.

4. Mice are generated from type I recombinant ES cells.

5. These mice are crossed to trangenic mice that express Cre recombinase in a tissue of interest. There it exises sequences between the two remaining loxP sites, inactivating the gene only in that tissue.

The new knockout vector is diagrammed in Figure 6, and is nearing completion. The various types of recombinant that will be generated, along with restriction fragment length polymorphisms that will be used to distinguish them, are diagrammed in Figure 7. I plan to express Cre recombinase in breast tissue of female trangenic mice by placing it under control of regulatory sequences for the whey acidic protein (WAP). WAP is a milk protein whose expression is restricted to the mammary gland (Pittius et al.,1988a), and is under hormonal control (Pittius et al.,1988b). The use of this promotor is advantageous for several reasons. Because it is expressed only in female mice, both the transgene and the knockout mutation can be transmitted simultaneously through males, allowing substantial savings in mouse husbandry costs. WAP sequences have been used successfully to target expression of transgenes to the mouse mammary gland (Tzeng et al., 1993, Jhappan et al., 1993). Most importantly, the differentiation of breast tissue has been studied extensively.

7. Conclusions

1. The human ASF/SF2 gene resides on human chromosome 17q21.3-22. It is distinct from the BRCA1 gene, although this observation does not exclude the possiblity that ASF/SF2 plays a role in the etiology of breast cancer by regulating the expression of BRCA1 or other growth control gene(s).

2. The ASF/SF2 gene is close, but distal to the *Ovum mutant* (*Om*) locus in mice.

3. Seven new embryonic stem cell lines have been isolated that are heterozygous for deletions of the ASF/SF2 gene. Five of these cell lines have been injected into blastocysts, and chimeric mice have been obtained from four lines. However, highly chimeric mice are runted with hypotrophic testes that lack germ cells, and are sterile. No germline transmission of the offspring have been observed. At the present time we cannot distinguish between a technical problem with the handling/injections of the ES cells and haploinsufficiency of the ASF/SF2 gene.

4. The ASF/SF2 gene is required for viability of a chicken B cell line (Wang and Manley, 1996), and may be required for the viability of other cell types as well. We have found that ASF/SF2 is widely expressed in mouse embryos. Therefore deletion of ASF/SF2 in all cells likely will result in early embryonic lethality.

5. A knockout vector that will allow tissue-specific deletion of the ASF/SF2 gene is nearing completion.

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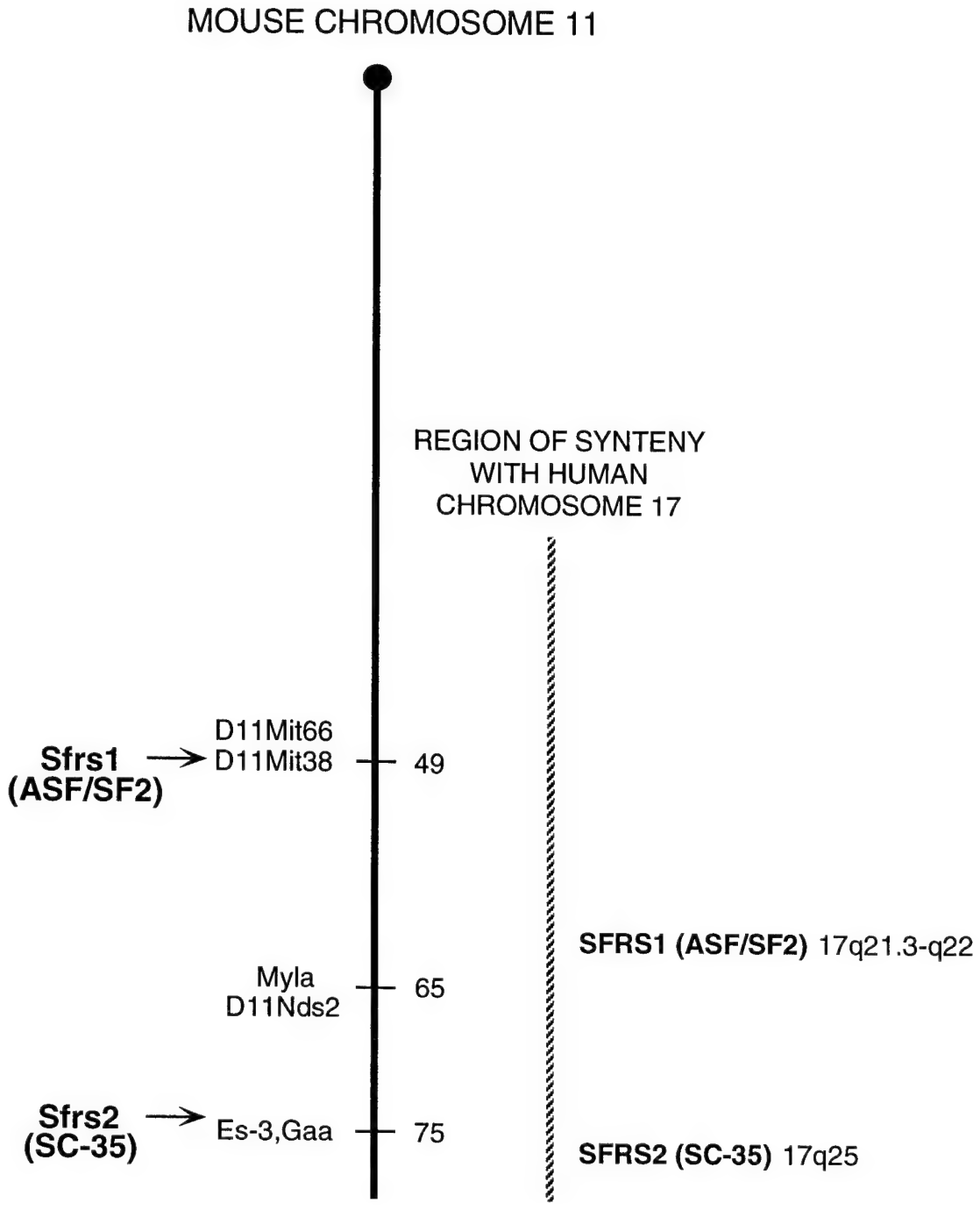
9. Appendix.

Figure 1. Chromosomal locations of the mouse and human ASF/SF2 and SC-35 genes.



Locations of the Sfrs1 (ASF/SF2) loci on mouse choromosome 11 and human chromosome 17q (Bermingham et al., 1995). Also shown are the loci of the mouse and human SC-35 genes, which encode a related SR protein.

Figure 2. Targeted mutagenesis vector for the ASF/SF2 gene.

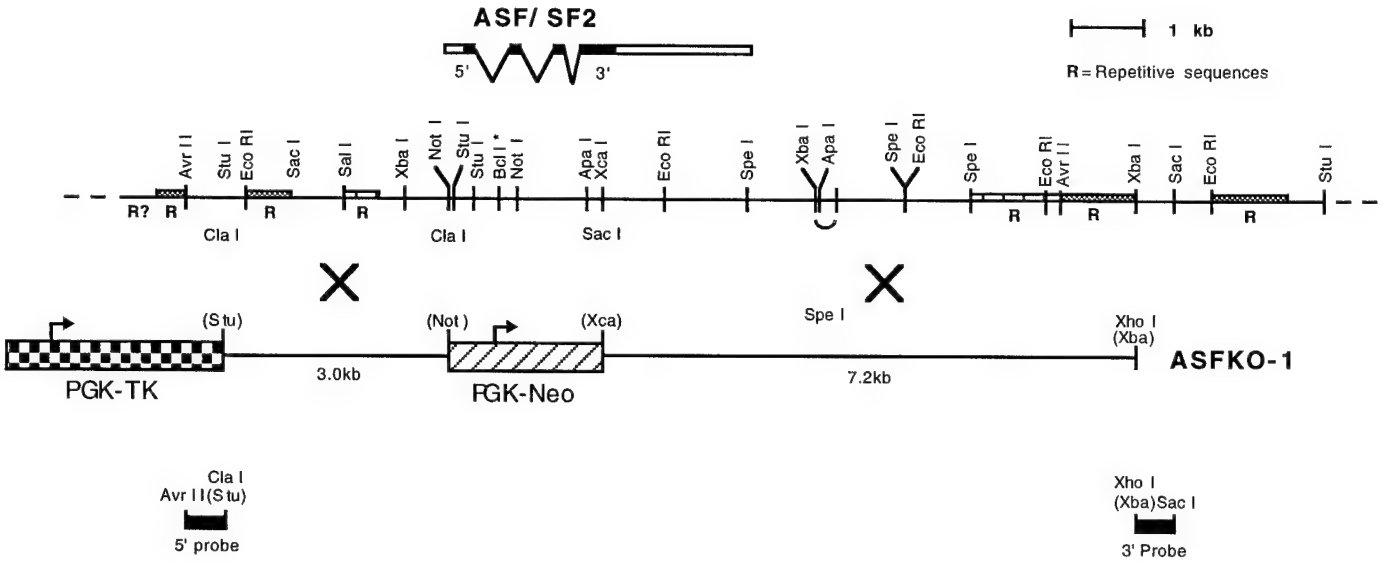
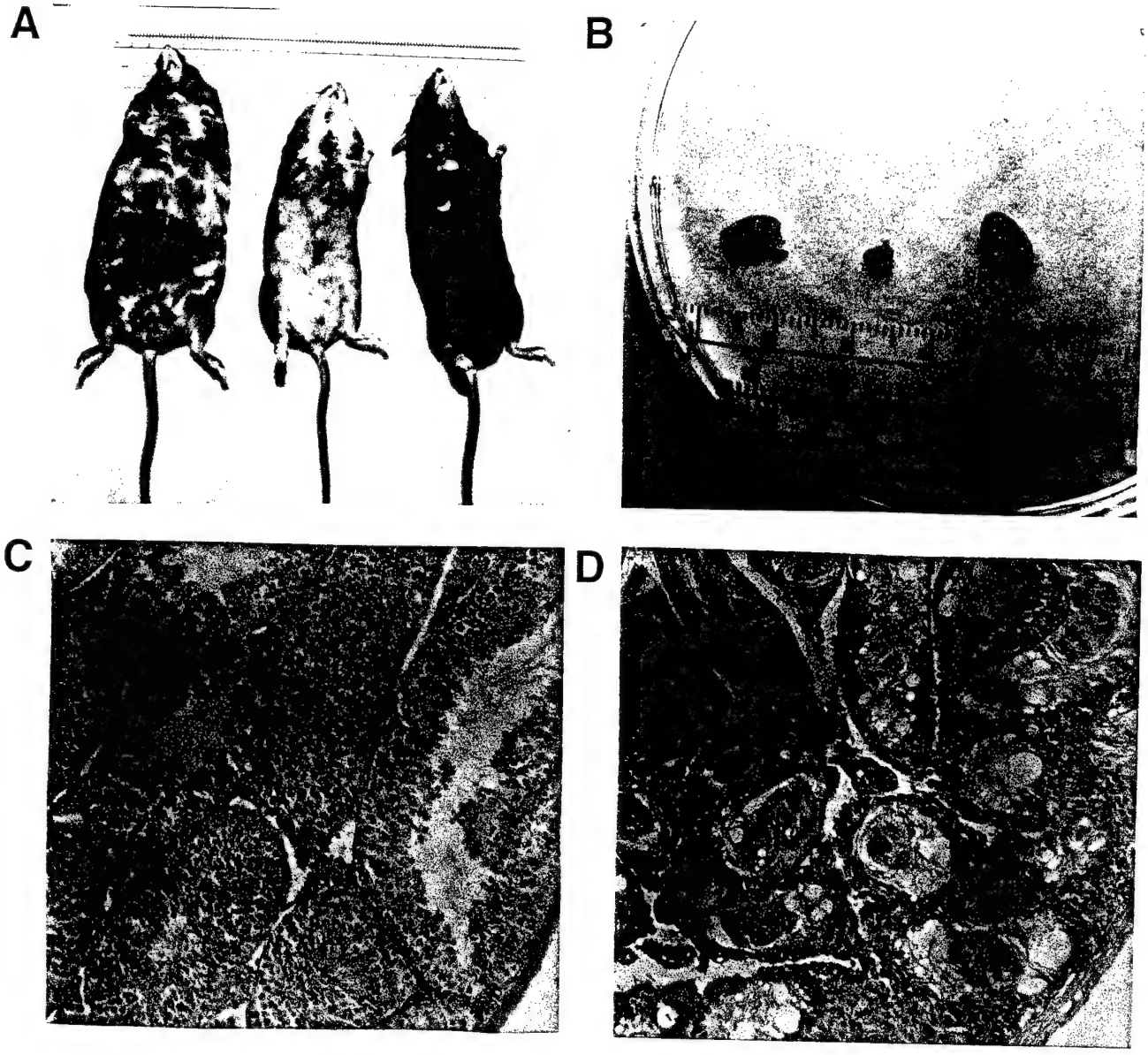


Figure 3. Phenotypes of chimeric mice that were generated from ES cells heterozygous for a deletion of the ASF/SF2 gene.



Legend: **A**. Examples of ASF/SF2 chimeric mice. Left: modestly chimeric, fertile male mouse. Center: highly chimeric, sterile male mouse. These mice were runted soon after birth, and never achieved the body weight of their littermates. Right: C57BL/6 control mouse. **B**. Testes from the mice shown in A. Testis from highly chimeric mouse is reduced in size compared to those from a fertile chimera, and control mice. PCR analysis of contralateral testes from the chimeras shown indicates that the fertile chimera possessed reduced proportions of cells that contained the neomycin resistance gene, compared to the sterile chimera. **C, D**. Testes shown in B. were fixed with Bouin's fixative, sectioned and stained with hematoxylin and eosin. Few, if any, germ cells can be observed in the testis from the highly chimeric mouse, D. Scale: 1cM = 90μ.

Figure 4. Template for mouse ASF/SF2 *in situ* hybridization probe.

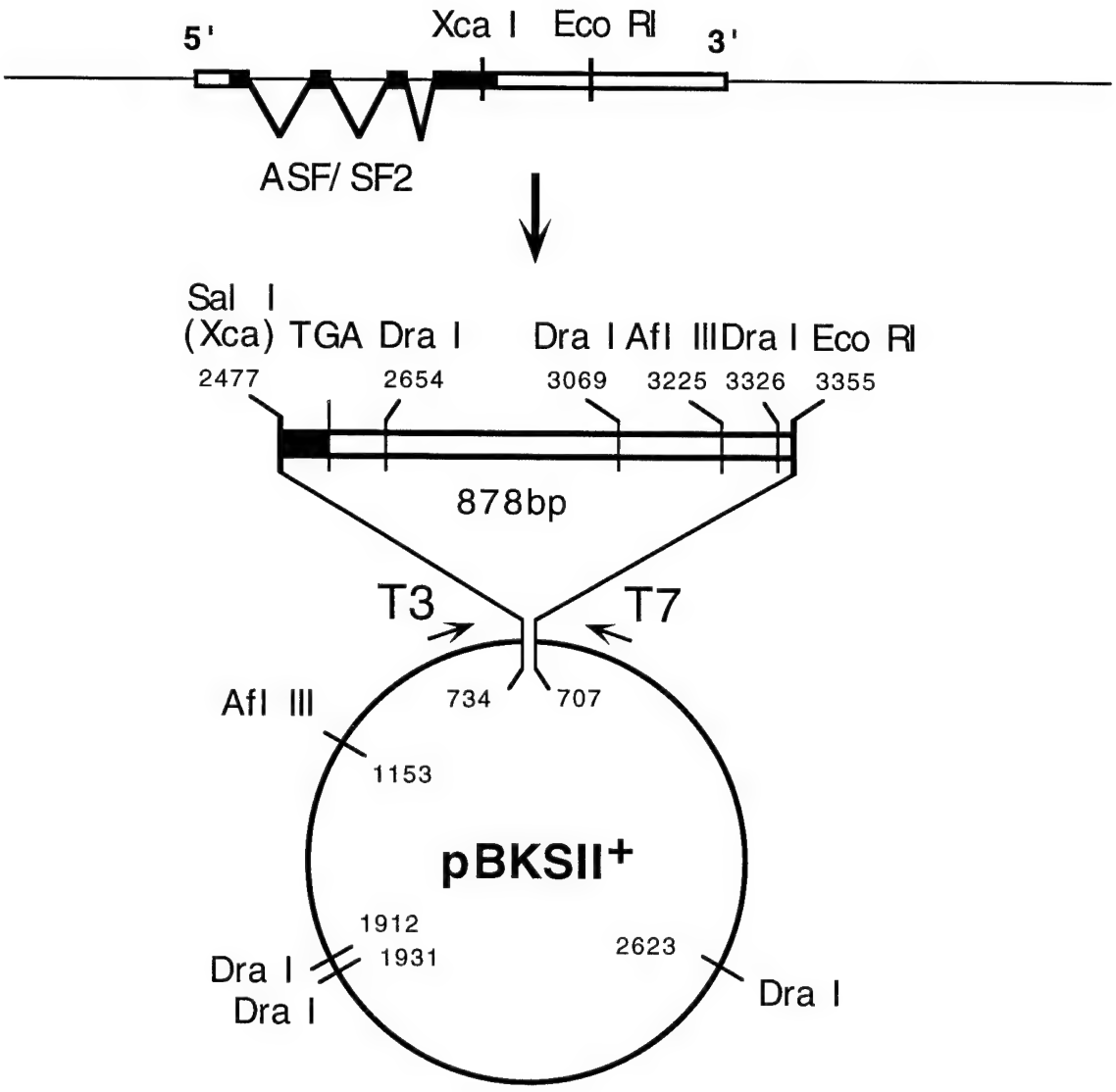


Figure 5. Strategy for tissue-specific gene deletion.

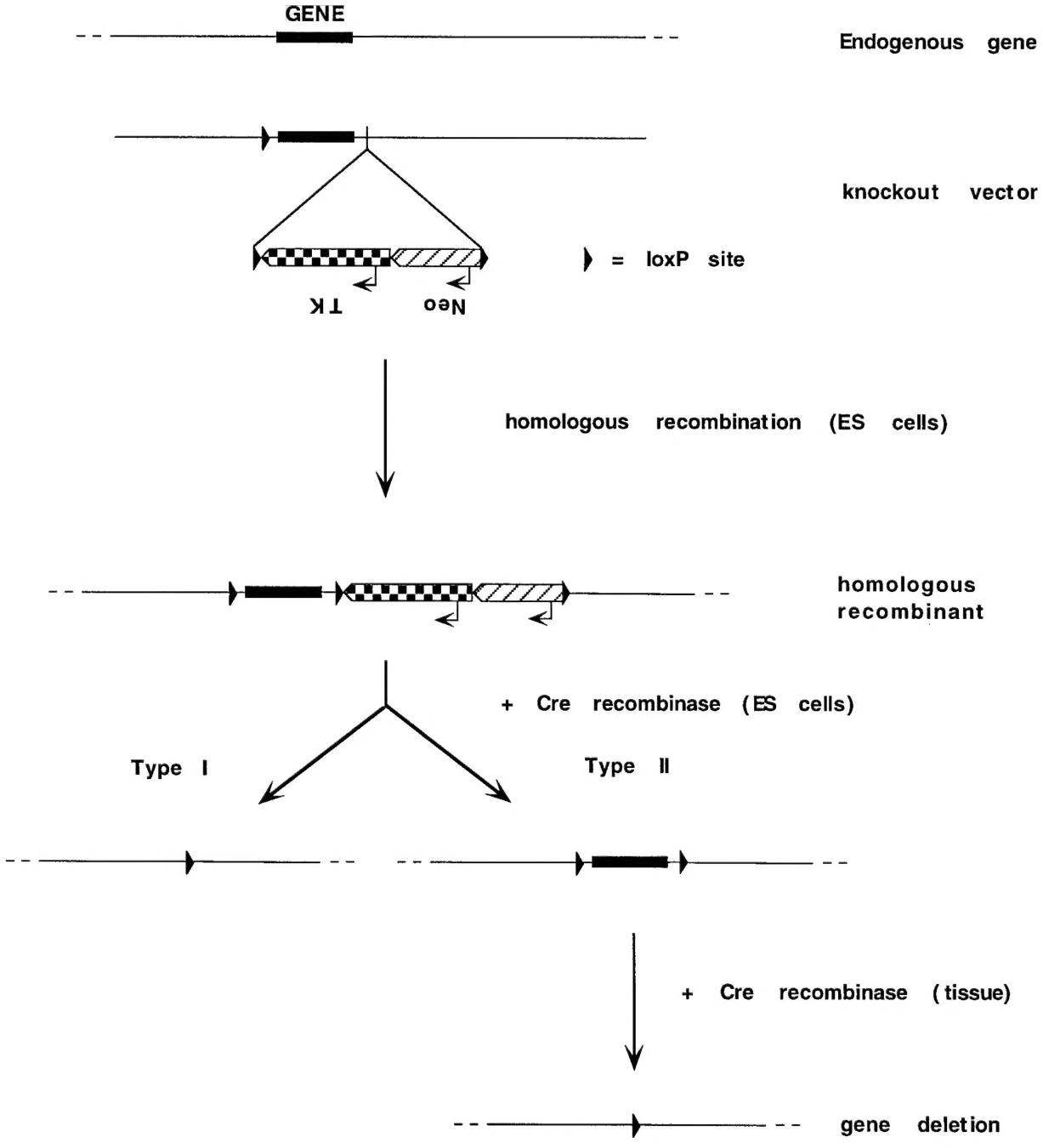


Figure 6. A vector for generating inducible deletions of the ASF/SF2 Locus.

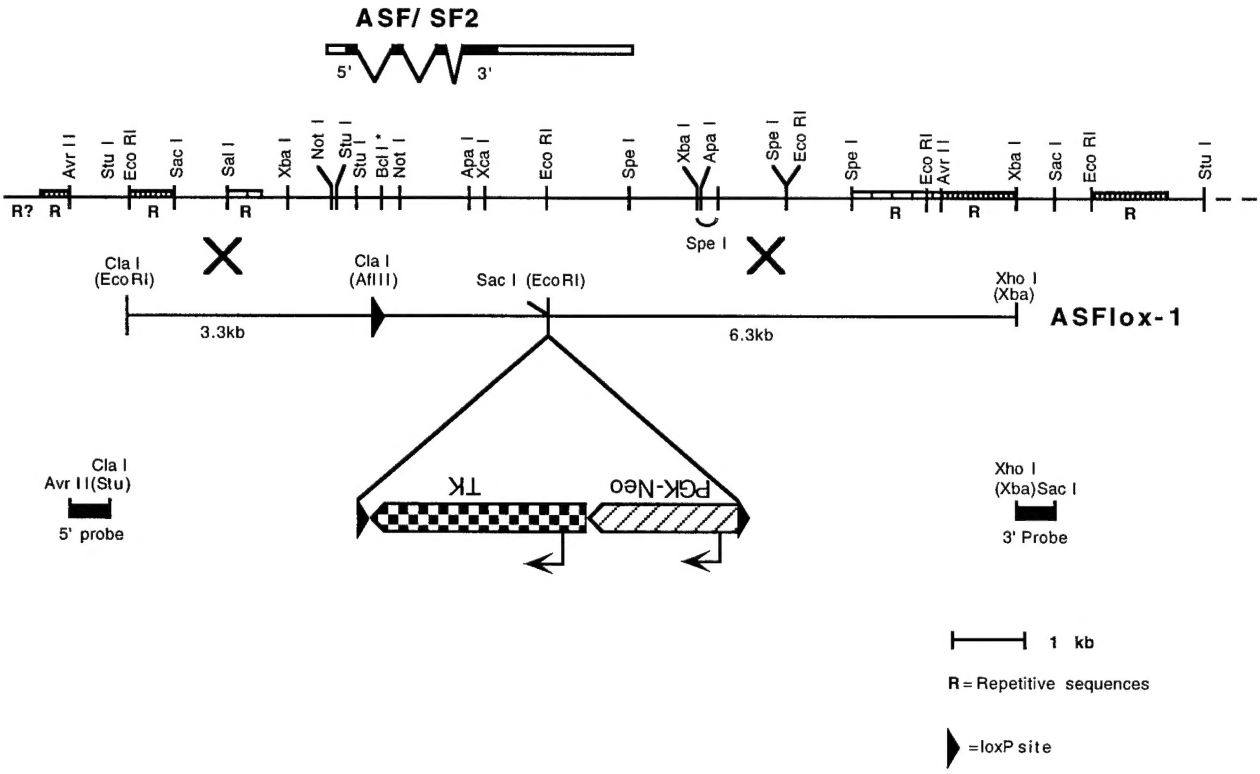


Figure 7. Restriction fragment length polymorphisms for mutations at the ASF/SF2 locus.

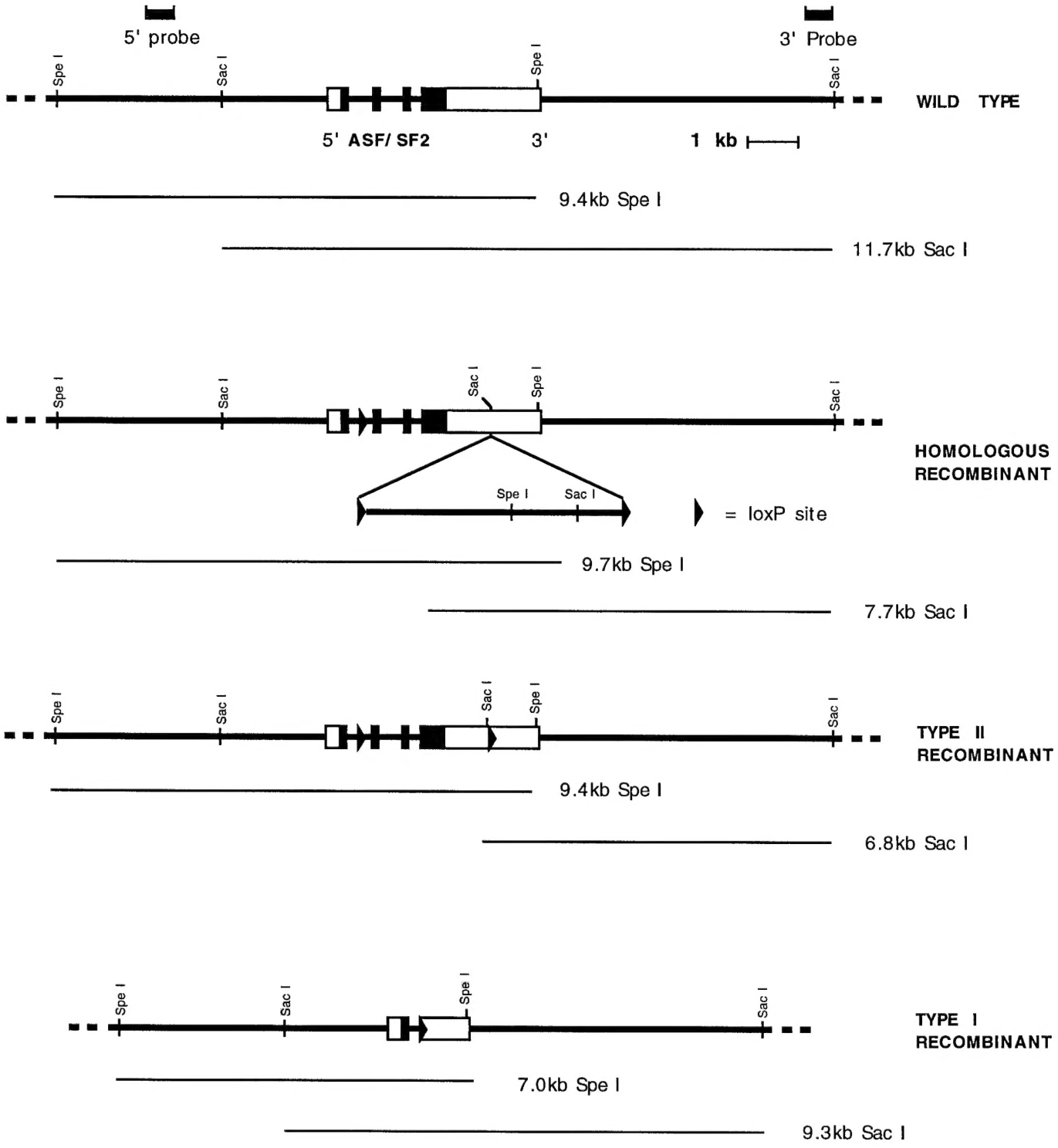


Table 1. Summary of Current ASF/SF2 Knockout Results.

	EXPERIMENT 1	EXPERIMENT 2	TOTAL
ES Cells			
# cell lines screened:	100	162	262
# ASF/SF2 +/- cell lines:	1	7	6 (2.3%)
# cell lines injected:	1	5	6
Mice			
#chimeric mice:	7	16	23
% chimerism:	20-60	20-80	
Sex ratio bias?	yes	yes	
Germline transmission?	no	no	